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Phylogenetic Position of Acoel Turbellarians Inferred from Partial 18S rDNA Sequences

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ABSTRACT—Primitive platyhelminths, especially Acoel turbellarians, are thought to be key to understanding the origin and evolution of metazoa. In order to infer their phylogenetic position within the phylum Platyhelminthes, we determined and compared the complete nucleotide sequence of a region of about 750 base pairs in the central part of an 18S rDNA for ten turbellarians, including two species of the group Acoela, six species of the group Polycladida, and two species of the group Tricladida. The deduced phylogenetic tree suggests that the three groups examined form discrete and separate entities. In addition, the tree suggests an earlier emergence of the Acoel turbellarians than the other platyhelminths. This animal may not be derived by means of secondary reduction from advanced acoelomates but may be nearest to its metazoan ancestors.

INTRODUCTION

The origin and evolution of multicellular animals (metazoans) have received considerable attention over the years [4, 9–11, 14, 26]. It is generally accepted that the metazoa arose from protozoa, perhaps 700–1000 million years ago. Models of the metazoic origin suggest that either colonial flagellates [10] or syncytial ciliates [9, 11] became acoel flatworm-like creatures (phylum Platyhelminthes), from which various modern metazoans have diverged. Current debate concerns whether the metazoa are monophyletic or polyphyletic and what the ancestral metazoa were like. Recent studies of the molecular phylogeny of invertebrates have suggested that the metazoa are polyphyletic (Cnidarians arose from a protist ancestry different from the Bilateria) [5, 8] and within the Bilateria, an early split gave rise to Platyhelminthes and the coelomate lineage [8, 13], although a monophyletic origin of the metazoa has also been proposed [16]. Regardless of route, primitive platyhelminths, especially Acoel turbellarians, are key animals in understanding the origin and evolution of modern metazoans.

In the present investigation, we compared molecular sequence data derived from 18S rDNAs to estimate the phylogenetic position of Acoel turbellarians. The 18S rRNA or its gene (18S rDNA) is ideally suited for phylogenetic studies of distantly related organisms because it is rich in information and the sequencing methodology permits the rapid accumulation of very large databases. According to Pace *et al.* [18], these molecules are conservative in overall structure and constitute a single gene family, so that problems of establishing homology among paralogous genes are avoided. The rRNA gene seems to be free of artifacts of lateral gene transfer between phylogenetically distant organisms. This and the absence of paralogous genes mean that 18S rRNA sequences accurately reflect phylogenetic relationships among the organisms from which the rRNA were prepared. The rRNA are present in large amounts in all organisms and are easily isolated. The conservative nature of the rRNA structure extends to the nucleotide sequence. Some regions of the molecule are highly conserved among distantly related species. Using these uni-

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versally conserved regions, direct rapid sequencing of 18S rRNA (DNA) was achieved. Enough data were accumulated from 18S rRNA sequences for statistically significant comparisons. Since each of the 1000 or so sequenced nucleotides constitutes a characteristic state, the evolutionary information content of rRNA sequences is very high.

Here we determined the complete nucleotide sequence of a region of about 750 base pairs (bp) in the central part of the 18S rDNA for ten turbellarian species consisting two species of Acoela, six of Polycladida and two of Tricladida. The phylogenetic relationship among these turbellarians was analyzed by sequence comparison. Our primary interest was in determining whether the acoel turbellarian is a primitive platyhelminth or a secondary reduction from advanced acoelomates.

MATERIALS AND METHODS

Animals

The nine turbellarian species examined included two species of the order Acoela, *Convoluta naikaiensis* (Yamasu) and *Amphiscolops* species (Yamasu), six species of the order Polycladida, *Notoplana koreana* (Kato), *Planocera multitentaculata* (Kato), *Stylochus orientalis* (Bock), *Pseudostylochus obscurus* (Stimpson), *Stylochoplana pusilla* (Bock), and *Thysanozoon brocchii* (Risso), and two species of the order Tricladida, *Dendrocoelopsis lactea* (Ichikawa et Okugawa) and *Dugesia japonica* (Yeri et Kaburaki). Whole animals were frozen in liquid nitrogen and kept at −80°C until use.

DNA isolation

Frozen and powdered samples were lysed in TE buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 8.0) that contained 0.5% sodium dodecyl sulfate. After digesting samples with proteinase K (100 μg/ml) at 50°C for 3 hr, DNA was extracted with phenol and precipitated in ethanol and an equal volume of 5.0 M ammonium acetate. Samples resuspended in TE buffer were further purified by RNase A (20 μg/ml) digestion at 37°C for 1 hr followed by ethanol precipitation.

Adults of *Convoluta naikaiensis* have symbiotic alga. To avoid contamination of algal DNA, that of the turbellaria was extracted from embryos.

Amplification of the central region of 18S rDNA and sequencing

A region of about 1000 bp from the central part of 18S rDNA was amplified by the polymerase chain reaction (PCR) [20] in a Perkin Elmer Cetus thermal cycler. Amplifications were performed in 100 μl of 50 mM KCl, 1.5 mM $MgCl_2$, Tris-HCl (10 mM, pH 9.0), 0.1% Triton X-100, with 0.2 mM each dNTP, 100 pM primer, template DNA (10–100 μg) and 2 U *Taq* DNA polymerase (Promega). Primer-1 [5′-CAG(CA)CCCGCGGTAAT(TA)C-3′] and primer-2 [5′-ACGGGCGGTGTGT(AG)C-3′], the latter being identical to Primer C of Field *et al.* [8], were used for amplification. One of the primers was kinased prior to PCR at the 5′ terminal phosphate. The temperature regimen for 30 cycles was 1 min at 92°C, 2 min at 55°C, and 3 min at 72°C.

According to the method described by Higuchi and Ochman [12], single-stranded DNA was obtained by digesting the amplified product with lambda exonuclease. The nucleotide sequence of the single-stranded PCR products was directly determined by dideoxy chain-termination [22] using Sequenase ver 2.0 (USB) and [^{35}S]-dATP (Amersham). In addition to primer-1 and -2, primer-3 (5′-TTGGCAAATGCTTTCGC-3′), primer-4 (antisense of primer-3), primer-5 [5′-ATTCCTTT(AG)AGTTTC-3′] and primer-6 (antisense of primer-5) were used in sequence determination.

Comparison of sequences and inferences about phylogeny

Sequences were aligned on the basis of maximum nucleotide similarity. Using the aligned sequences, evolutionary distance values were calculated pairwise as described by Jukes and Cantor [15]. The phylogenetic tree was inferred from an analysis of results by the neighbor-joining method of Saitou and Nei [21]. The degree of support for internal branches of the tree was further assessed by bootstrapping [7].

The corresponding sequences of *Saccharomyces*

cerevisiae [17] and *Neurospora crassa* [17] provided data of outgroup organisms.

RESULTS

Partial nucleotide sequences of the 18S rDNAs from ten turbellarians

The complete nucleotide sequences of a region of about 750 bp in the central part of the 18S rDNA of ten species of turbellarians are summarized in Figure 1. The sequences correspond to positions 910–1646 in the sequence of human 18S rRNA [17]. Alignment of the nucleotide sequences from the ten planarian species revealed some key features. In some regions, the nucleotide sequences were highly conserved. For example, among the ten species, few changes were evident in the sequences from positions 277 to 380. In contrast, the nucleotide sequences of other regions, such as positions 214–267 and 543–614, were highly variable. Sequences of yet another region varied only moderately. These regional differences may reflect differences in the functional constraints upon the regions in the 18S rRNA.

The sequence alignment showed that the six species of the order Polycladida share common nucleotide sequences, as do the two species of the order Tricladida. However, the nucleotide sequences of these two groups are very different, suggesting their discrete separation. In addition, the nucleotide sequence of the two acoela species *Convoluta* and *Amphiscolops* differed from those of Polycladida and Tricladida so much that almost the entire region of the 18S rDNA we examined was changed. This suggests a large evolutionary divergence of the acoel turbellarians from the two other groups of planarians.

Phylogenetic relationships among the ten species of turbellarians

Structural similarity and evolutionary distance values were calculated pairwise as described [15] between the sequences aligned in Figure 1. The results are summarized in Table 1. A phylogenetic tree was constructed by the neighbor-joining method [21], by reference to the distance values of Table 1. The phylogenetic tree shown in Figure 2 indicated that the ten turbellarians can be subdivided into three groups, which correspond to the orders Acoela, Polycladida and Tricladida, and that the acoel group emerged first among the three. The internal branches of the tree were supported by the high bootstrapping values. Among the six species of Polycladida, the branching of *Thysanozoon* belonging to suborder Cotylea from the five species of Acotylea was evident (Fig. 2).

The phylum Platyhelminthes is subdivided into four major groups, Turbellaria (planarians), Monogenea (monogenetic flukes), Trematoda (digenetic flukes), and Cestoda (tapeworms) [4, 14]. Recent studies of the phylogenetic relationships among several parasitic platyhelminths by comparison of partial 18S rRNA sequences [2, 17] support the hypothesis that platyhelminths are monophyletic; that planarians emerged first, then flukes and finally tapeworms evolved. Together with the results of the present study, we constructed a phylogenetic tree to examine the position of acoel turbellarians within the phylum Platyhelminthes (data not shown). The tree suggests that *Convoluta* and *Amphiscolops* are more primitive members within Platyhelminthes.

Phylogenetic position of acoel turbellarians in the animal kingdom

The complete or partial nucleotide sequences of 18S rRNA (or DNA) of various phyla, including *Artemia salina* [17], *Caenorhabditis elegans* [17], *Ciona savignyi* [19], *Xenopus laevis* [17], and *Homo sapiens* [17] have been reported. The phylogenetic tree (Fig. 3) shows our positioning of the Acoel turbellarians, Polycladida and Tricladida in the animal kingdom. It is evident that these three groups of turbellarians are not interrupted by any other animal groups. Also, the branching point of the acoel turbellarians is earlier than those of the other turbellarians compared in this study.

DISCUSSION

In this investigation, we determined and compared the complete nucleotide sequence of a region of about 750 bp in the central portion of the 18S rDNA from ten species of turbellarians. The

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                    ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,...70
 1. Stylochus       TTGTTGGTTTTCGGAACA-----TGAAGTAATGATTAAGAG---GGACAGAC-GGGGGCATTCGTATTGC
 2. Planocera             -
 3. Pseudostylochus
 4. Notoplana
 5. Stylochoplana
 6. Thysanozoon                                  C
 7. Dendrocoelopsis                 -                      A     A   T C          AT    GCT
 8. Dugesia                       A   -                    A     A     C-         AT    GCT
 9. Convoluta             AAC CGG G CTGTT    GCTCG  G      G A      A    G          T
10. Amphiscolops        A C      C TACCG AGTTTCCCTCAG  T GC  A  CTT A    G A        TC

    ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,..160
 1. -GGTGGG-AGAGGTGAAATTCTTGGATCATCGCAAGAC-GCCCTAC---AGCGAAAGCATTTGCCAAGAATGTTTCCATTAATCAAGAAC
 2.                                        -
 3.
 4.
 5.
 6.                      A               G  A
 7.      T              A A       AGC     AAA       T                          T
 8.      CT              GA  C    AGC    GAAA       T                          T
 9.  TAC TT                    G G        AAA       T                          T
10.  TAG TTC                   C A        AAA    TACT                          T

    ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,..250
 1. GAAAGTCAGAGGTTCGAAGACGATCAGATACCGTCCTAGTTCTGACCATAAACGATGCCAACTGGCAA-TCCGTTGCGATTGCAAGTTCG
 2.                                                                                    G
 3.
 4.
 5.
 6.                                                        CTG    C CG
 7.            A                                          T          A  G  TA CGAA G AATT CAATA
 8.       T    A                                          T      T   A G G TA CGAA GA ATT-TAATC
 9.           TA TA    G   T       C  G  A    A          TT A      TA G   TT  CC TG CC --TC
10.           TA T     G   T       C  G  A       T      T  A      TA G  CTT CCAAAG CAT- CCAT

    ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,..340
 1. ATCCAACGGGCAGCCT-CCGGGAAACCAAAGTCTTTGGGTTCCGGGGGAAGT-ATGGTTGCAAAGCTGAAACTTAAAGGAATTGAC-GGA
 2.                                                                                    T
 3.
 4.
 5.
 6.                                  AT
 7. TC TTG TA A  T A                 A
 8.    TTG TA A  T A                 A                G
 9.  CTGGG AA A TTTAA                T A T                        ATC
10.  CTTGG AA A  TTAA                T A T                        AT

    ....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,....+....,..430
 1. AAGGCACCACAAGGAGTGGAGCCTGCG-CTTAATTTGACTCAACACGGGAAAACTCTACCCGGCCCGGACACTGTGAGGATTGACAGATT
 2.       -                 G                          -
 3.                                                    -
 4.                         G                          -
 5.                -                                   -
 6.                -   -  - C                          -          -      G  T
 7.  G        C                                    T    -      T
 8.  G        C       -   -   -                    TT   -C     T
 9.  G        C                                  G      -   A  T  A    TCT A              A
10.  G        C                                         -   G  T  A    TCT A
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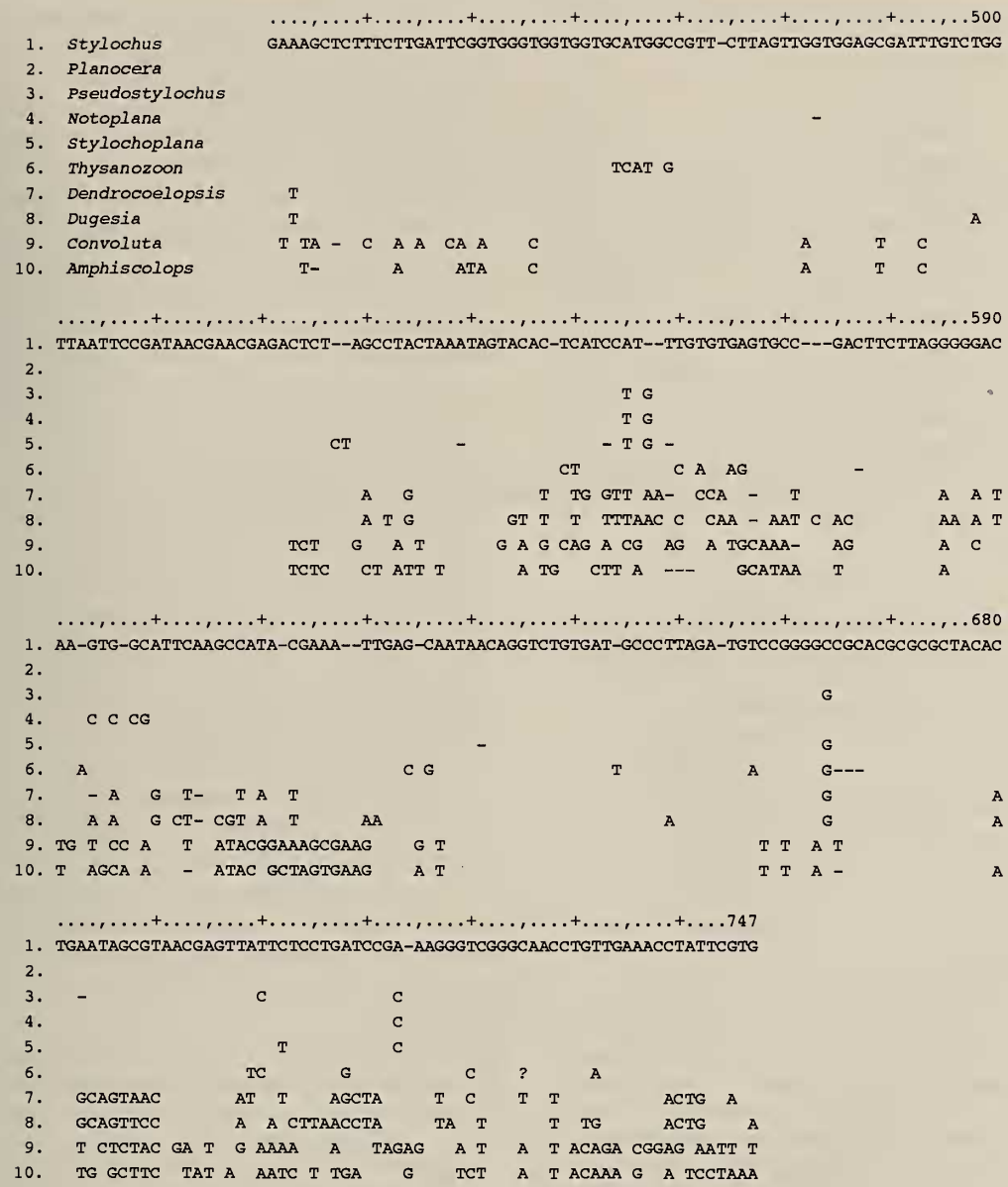

FIG. 1. Alignment of the central regions of the 18S rDNAs from ten Turbellarians. All the bases are shown for *Stylochus orientalis* and only bases different from these are shown for other species. Bars indicate deletions.

TABLE 1. Structural similarity and evolutionary distance data for turbellarian 18S rDNA sequences

Genera	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
1. *Stylochus*		0.000	0.004	0.002	0.005	0.048	0.100	0.131	0.221	0.214	0.184	0.188
2. *Planocera*			0.004	0.002	0.005	0.048	0.100	0.131	0.221	0.214	0.184	0.188
3 *Pseudostylochus*				0.002	0.002	0.048	0.100	0.131	0.223	0.219	0.188	0.191
4. *Notoplana*					0.004	0.049	0.102	0.134	0.221	0.216	0.186	0.188
5. *Stylochoplana*						0.049	0.098	0.134	0.223	0.216	0.188	0.188
6. *Thysanozoon*							0.134	0.170	0.270	0.248	0.228	0.231
7. *Dendrocoelopsis*								0.049	0.231	0.209	0.177	0.173
8. *Dugesia*									0.250	0.228	0.205	0.200
9. *Convoluta*										0.117	0.236	0.245
10. *Amphiscolops*											0.233	0.238
11. *Saccharomyces*												0.067
12. *Neurospora*												

The values are average numbers of nucleotide substitutions per sequence position determined by the Jukes and Cantor formula [15].

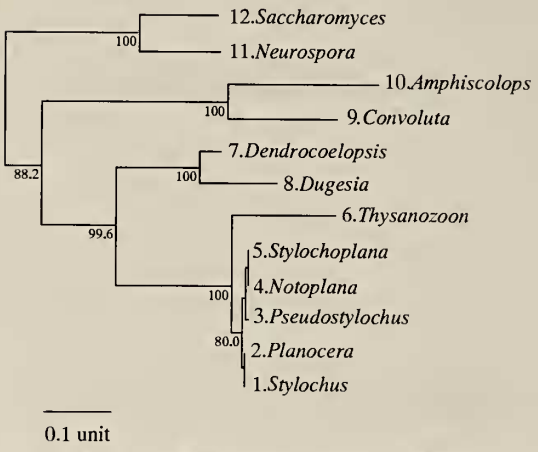

FIG. 2. The phylogenetic tree of ten turbellarians, as deduced by the neighbour-joining method using the fraction of observed substitution difference over all sites. The scale bar indicates an evolutionary distance of 0.1 nucleotide substitution per sequence position. Numbers at each branch indicate the percentage of times a node was supported in 500 bootstrap pseudoreplications by the neighbour-joining method.

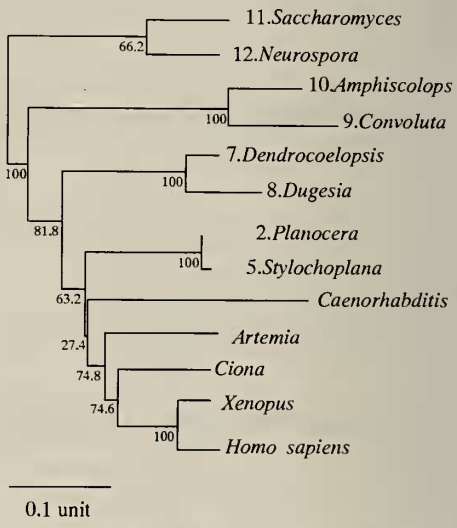

FIG. 3. Phylogenetic relationships of the turbellarians with other organisms (*Neurospora crassa, Saccharomyces cerevisiae, Caenorhabditis elegans, Artemia salina, Ciona savignyi, Xenopus laevis* and *Homo sapiens*). The tree was constructed by the neighbour-joining method. The scale bar indicates an evolutionary distance of 0.1 nucleotide substitution per sequence position. Numbers at each branch indicate the percentage of times a node was supported in 500 bootstrap pseudoreplications by the neighbour-joining method.

phylogenetic tree (Fig. 2) indicated that the ten species can be subdivided into three groups, which correspond to the orders Acoela, Polycladida and Tricladida. In addition, together with the phylogenetic tree including other groups of the animal kingdom (Fig. 3), early emergence of the acoel turbellarians in metazoic evolution is evident. Therefore, they might be some of the closest multicellular animals to their metazoan ancestors. The early emergence of acoel turbellarians will be substantiated further by investigating with other

molecular probes such as 28S rDNA.

A phylogenetic tree constructed from the 5S rRNA sequences suggested that the branching points of *Dugesia japonica* (Tricladida) and nematodes (eg, *Caenorhabditis elegans*) are slightly earlier than those of other metazoans, including *Planocera reticulata* (Polycladida) [9]. That means turbellarians including Tricladida and Polycladida are polyphyletic. However, that view was not supported in the present study (see Figs. 2 and 3). Even when nematodes are included in the 18S rRNA (DNA) tree (Fig. 3), Tricladida and Polycladida are categorized into one larger group, or Turbellarians.

It seems a consensus that all platyhelminths except for the lower turbellarians, including Acoela, Catenulida and Nemertodermatida, are of monophyletic origin [1–3, 6, 23, 24]. However, the relationships among the Catenulida, the Nemertodermatida-Acoela and the other orders of Platyhelminthes, are problematic [1, 3, 6, 23, 24]. Most views of the relationships within the Platyhelminthes support the hypothesis that a primary acoelomate condition is derived from an acoel-type worm, and as the phylum evolved, the gut became more elaborate and the division into distinct germ layers more pronounced. The Acoela, which are virtually solid with a ventral mouth and central 'syncytial' area acting as the gut, have many primitive features, and have been considered nearest to the ancestral group within the phylum [11]. Catenulida and Nemertodermatida, with fully a developed gut and more distinct mesodermal matrices, are assumed to represent the next level of sophistication. Recently, however, several investigators have argued that Catenulida and Nemertodermatida, the latter, in particular, may be more primitive [24, 26]. Acoela are unusual in having modified cilia, in lacking any intercellular matrix [24], and in showing a peculiar form of 'duet' cleavage similar to that of gastrotrichs and nematodes (cf. [26]). They might therefore be a derived group, an example of secondary reduction. The present 18S rDNA sequence analysis, however, did not support this view, namely the branching point of *Convoluta* was much earlier than those of other turbellarians. Further studies of the molecular phylogeny of Catenulida and Nemertodermatida will offer more conclusive evidence of turbellarian evolution.

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